A STUDY OF SOME METHODS FOR THE ANALYSIS OF PHENOLS AND RELATED SUBSTANCES WITH SPECIAL REFERENCE TO COULOMETRY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Lund by 1978

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This review summarizes and discusses results from the following papers which will be referred to by the Roman letters I - VII.

I B. Kinberger, L.-E. Edholm, O. Nilsson and B.E.F. Smith. A General Method for Coulometric Titration of Alkylphenols with Bromine. Talanta, 22 (1975) 979.

II B. Kinberger, L.-E. Edholm and B.E.F. Smith. Coulometric Titration of Deactivated Phenols with Bromine. Talanta, 22 (1975) 1042.

III L.-E. Edholm. A Coulometric Method for the Assay of Some Easily Oxidized Organic Substances with Iodine as an Oxidizing Agent. Talanta, 23 (1976) 709.

IV K. Callmer, L.-E. Edholm and B.E.F. Smith. A Study of Retention Behaviour of Alkylphenols in Straight and Reversed-Phase Liquid Chromatography and Application to the Analysis of Complex Phenolic Mixtures in Conjunction with Gas Chromatography. J. Chromatogr., 136 (1977) 45.

V L.-E. Edholm and K. Callmer. A Liquid Chromatographic Study of Brominated Alkylphenols and Investigation of Product Formation in the Coulometric Bromination of Some Alkylphenols. J. Chromatogr., 137 (1977) 363.

VI C. Hansson, L.-E. Edholm, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren. The Quantitative Determination of 5-S-Cysteinyldopa and Dopa in Normal Serum and in Serum from Patients with Malignant Melanoma by means of High Performance Liquid Chromatography. Clin. Chim. Acta. Submitted for publication.

VII L.-E. Edholm, C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren. Analysis of Cysteinyldopas, Dopa, Dopamine, Noradrenaline and Adrenaline in Serum and Urine Using High Performance Liquid Chromatography and Electrochemical Detection. J. Chromatogr. Submitted for publication.

INTRODUCTION

Phenols are defined as compounds containing one or several hydroxyl groups directly attached to an aromatic nucleus. They are of great industrial importance both as such and as starting materials for the preparation of various other organic compounds and of plastics. Simple derivatives are *e.g.* chlorophenols which have been found to exhibit outstanding germicidal and fungicidal properties and are used as fungicides and wood preservatives. Some chlorophenols are also important intermediates in the preparation of bactericides, germicides and plant-growth regulators[1].

Phenols also occur in living matter both in plants and animals. Thus, they are building stones in the naturally occurring polymers tannins and lignins and many phenols of less complex structure, like the flavanoids, are common in the vegetable kingdom[2]. Of special interest among phenols in the animal kingdom are those related to catechol. Catechol is the trivial name of 1,2-dihydroxyphenol. Biologically important catechols are derived from the amino acid tyrosine (see Fig. 1).

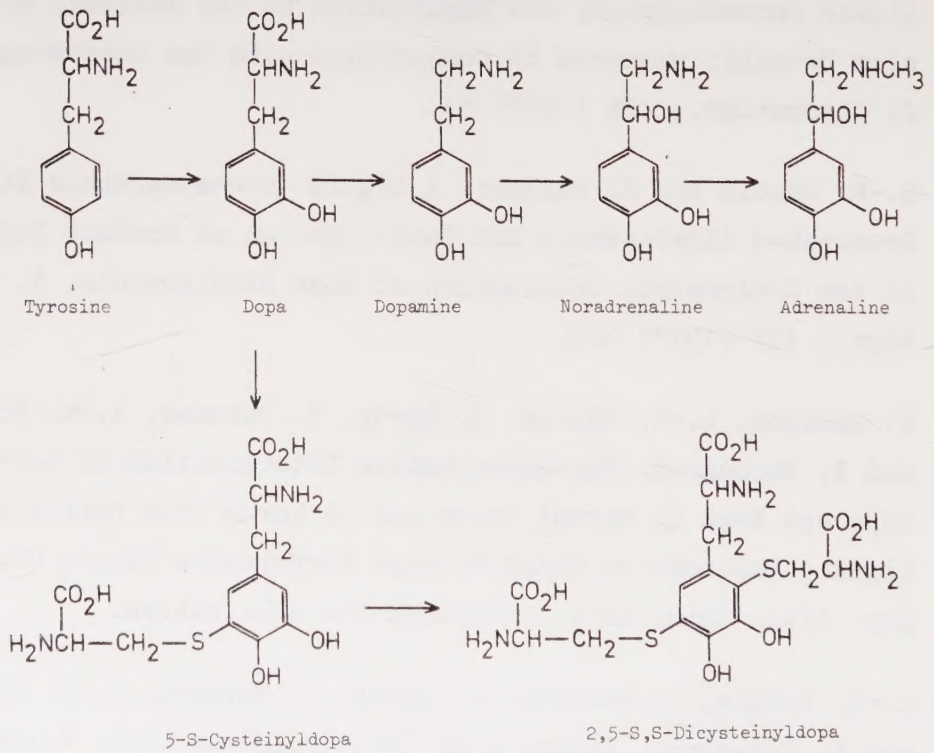


Fig. 1. The biochemical pathways for the formation of cysteinyldopas and catecholamines from tyrosine.

The catecholamines dopamine, noradrenaline and adrenaline are widely distributed in the animal kingdom. The amines are mainly found in adrenergic nerves in the central and peripheral nervous systems, in the adrenal medulla and in various endocrine organs. Recently several cysteinyl-substituted catecholic amino acids have been discovered. Thus, 2-S-, 5-S-, 6-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa have all been identified in urine from melanoma patients[3,4]. 5-S-Cysteinyldopa has been detected in serum[5] (VI, VII) and urine[6] (VII) from both normal subjects and patients with melanoma. In the latter case the concentration is raised. 2-S-Cysteinyldopa and 2,5-S,S-dicysteinyldopa were recently discovered in serum from a patient with melanoma (VII). The cysteinyldopas are formed in the tyrosinase-containing melanocytes and are the precursors to phaeomelanins.

The qualitative and quantitative analysis of phenols has since long been the subject of a vast number of investigations[7,8,9]. It is hardly an exaggeration to state that the bulk of existing analytical methods have been applied to the analysis of phenols in the course of the time. The methods used can conveniently be divided into three main groups, namely

(i) Methods for the determination of the total amount of phenols present in a sample;
(ii) Methods for the determination of separate phenols or a small number of phenols in a sample;
(iii) Methods for the simultaneous separation and quantitative determination of several phenols in a sample.

The methods studied in this work belong to group (ii) and (iii). Thus, "Coulometric titration of phenols with bromine (I, II) and "Coulometric assay of easily oxidized phenols with iodine as an oxidizing agent" (III) belong to group (ii) while "Chromatographic studies of alkylphenols and brominated alkylphenols" (IV, V) belong to group (iii) as do the investigations regarding "Determination of biologically important catechols" (VI, VII).

COULOMETRIC TITRATION OF PHENOLS WITH BROMINE (I, II)

This first part of the thesis includes two papers. The first one deals with the coulometric titration of alkylphenols with bromine (I) and the second one with the coulometric titration of deactivated phenols with bromine (II).

Coulometric analysis

Coulometric analysis is a designation of an analytical technique in which the quantity of electricity is employed to determine the amount of chemical substance in solution.

Coulometric analysis can be performed in two different ways. In the first the potential of a working electrode is maintained at some fixed value and the completion of the analysis is indicated when the current flow approaches zero.

In the second technique a constant current is used for the production of a reagent and is passed until an indicator signals completion of the reaction. The quantity of electricity passed to reach the end-point is calculated from the magnitude of the current and the time required. This method is also called coulometric titration.

To calculate the amount of analyte Faraday's law, which states that "The amount of chemical change produced by an electric current is proportional to the quantity of electricity passed" can be used. If 100 per cent current efficiency is obtained then Faraday's law can be expressed by the following equation. For a constant current

$$m = \frac{t \cdot I \cdot E \cdot 1000}{F} \qquad (1)$$

m = the weight of substance in µg
E = equivalent weight of substance
I = generating current in mA
t = time in sec
F = Faraday's constant = 96487 C/eq

Here coulometric analysis has been used for the titration of alkylphenols and deactivated phenols with coulometrically generated bromine. The equipment used is assembled from inexpensive standard pieces of apparatus thus making the method generally accessible.

Fig. 2 shows the coulometric cell used in (I) and (II). Fig. 3 shows a typical titration curve obtained in the coulometric titration used here.

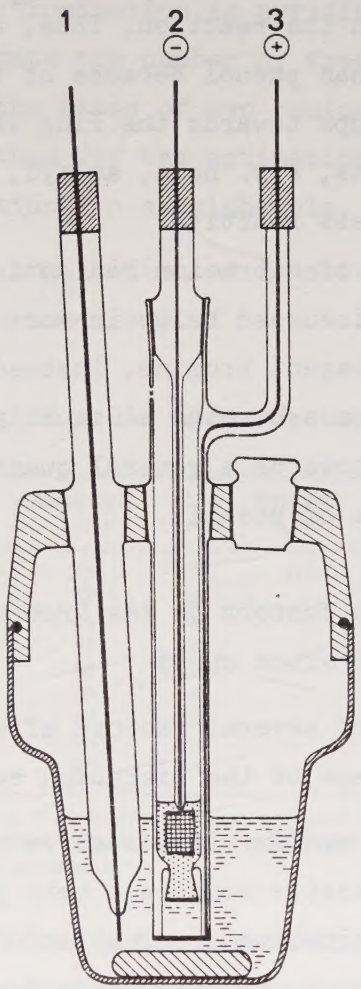


Fig. 2. Cell for coulometric titration. 1 = indicating electrode; 2 = cathode containing 2 M sulphuric acid; 3 = generating electrode (anode).

Bromination of phenols

The easy reaction between phenols and the electrophilic reagent bromine is the basis of the quantitative titration of phenols with this reagent[10,11,12]. The dominating influence in the bromination of phenols is excerted by the hydroxyl group whose well-known activation of the aromatic ring towards electrophilic reagents causes bromine to replace hydrogen at the free *ortho* and *para* positions. Other substituents in the phenol only have a modifying influence on the reaction. Thus, alkylphenols generally are more reactive than phenol because of the electron releasing effect of alkyl groups towards the ring while phenols with deactivating substituents, *e.g.* halo, acetyl, formyl, carboxyl and nitro groups, are less reactive.

However, the choice of titration medium is of decisive importance for the result as discussed below in more detail. The coulometric generation of the reagent bromine, instead of delivering it, or bromide-bromate, from a burette as usually done, means also a great step forward towards a general quantitative bromination method for all kinds of phenols.

Influence of various factors on the bromination reaction and on the shape of the titration curve

A systematic study of several factors affecting the bromination reaction and the shape of the titration curve was undertaken.

Choice of titration medium. It was already shown some 20 years ago[12] that aqueous acetic acid is a very good titration medium, permitting the quantitative bromination of a great number of phenols with good accuracy. However, in order to get quantitative results the titration conditions had to be adapted to the type of phenol and accordingly frequently changed. The present investigations were undertaken in order to develop a more uniform method based on aqueous acetic acid as titration medium and with coulometric generation of bromine.

The reactivity-promoting properties of the titration medium were governed by (i) the ratio of acetic acid to water, (ii) the concentration of bromide ion and (iii) the presence of pyridine (see Table 1). By proper choice of reaction medium alkylphenols can be titrated quantitatively either to the monobromination stage (without pyridine) or, in case of several vacant *ortho* and *para* positions, also to the full bromination stage (with pyridine). The fact that monobromination takes place predominantly in pyridine-free media and full bromination in pyridine-containing media makes it possible to estimate the number of free *ortho* and *para* positions in alkylphenols on the basis of two coulometric titrations. This is a new and simple method for the estimation of the number of free *ortho* and *para* positions in alkylphenols.

For the coulometric bromination of deactivated phenols a pyridine-containing medium is necessary.

Table 1. Composition of media used for bromination of phenols.

Medium	Acetic acid, % v/v	Water, % v/v	Pyridine, % v/v	$[Br^-]$, *M*
I-1	55	35	10	0·1
II-1	55	40	5	0·1
II-2	55	40	5	0·4
II-3	55	40	5	1·2
III-1	60	40	—	0·1
III-2	60	40	—	0·4
III-3	60	40	—	1·2
IV-1	90	10	—	0·1*
IV-3	90	10	—	1·2*

* In these cases lithium bromide was used instead of sodium bromide because of the low solubility of the latter in 90% v/v acetic acid.

Generating current. Ideally the generating current should have such a value that bromine is formed with the same speed as it is consumed in the substitution reaction. In that way side reactions due to bromine excess such as oxidation and bromine substitution in side-chains are avoided. On the other hand, at low currents the analysis time is too long and side-reactions of an unknown nature can cause erroneous results. A current of 3 mA was found suitable when determining phenols in amounts in the range 300 - 2000 µg.

Amount of phenol. The amount of phenol titrated is not insignificant. There is in fact a lower as well as an upper limit. The lower limit is set partly by the fact that a certain minimum distance is needed on the recorder chart in order to get an accurate measure of the time (see Fig. 3).

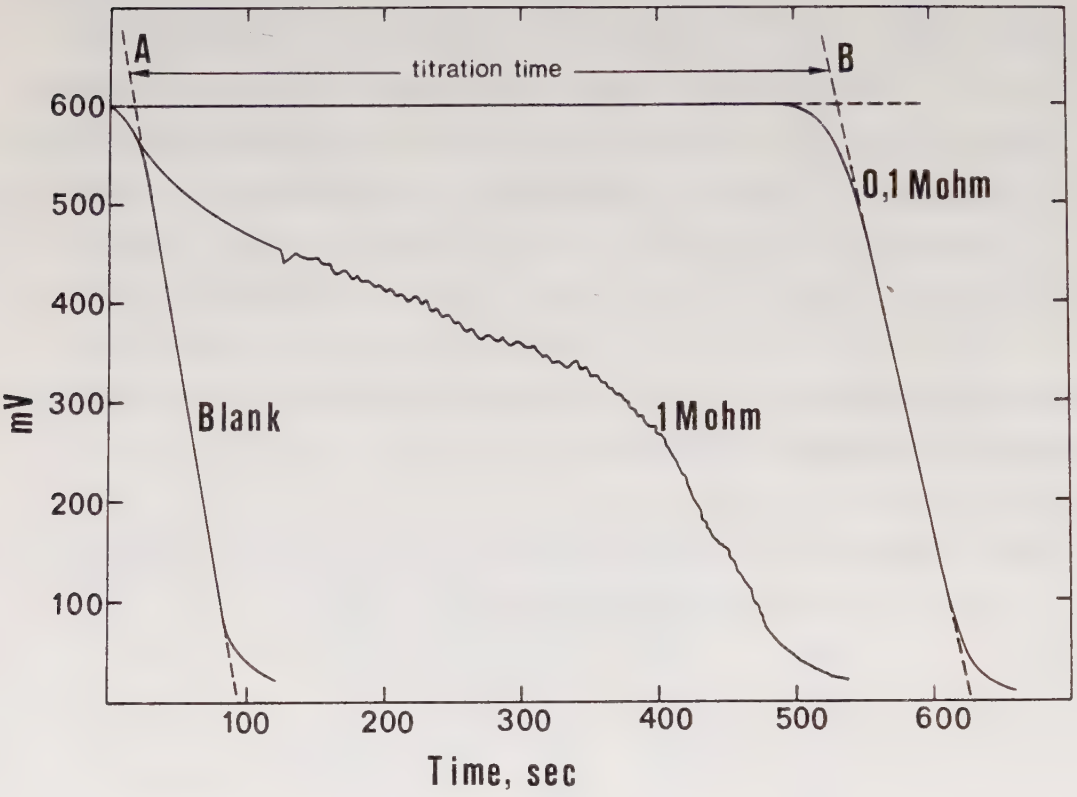


Fig. 3. Method of evaluating the end-point, and the dependence of the shape of the titration curve on the size of the polarizing resistance.

The lower limit is also influenced by a lack of correspondence between the rates of bromine generation and consumption.

The upper limit for the amount of phenol is partly determined by certain side-reactions at the generator anode and partly by a contamination of the indicating electrode. These reactions seem to start after a certain time of electrolysis and cause a relative error which increases with the analysis time *i.e.* the amount of phenol. In this investigation the exact li-

mits have not been settled but it is recommended that the amount of phenol titrated should consume between 10 and 20 µeq of bromine.

Polarizing resistance. The titration was followed by means of an indicating double platinum point electrode connected to the polarizing voltage terminal of a pH-meter through a polarizing resistance (see Fig. 4). The magnitude of this resistance has a decisive influence on the appearance of the titration curve (see Fig. 3). As seen, a resistance of 100 kΩ gives the most rapid depolarization, permitting an accurate evaluation of the end-point. It should be noted that the size of the polarizing resistance (100 kΩ) should be adapted to the magnitude of the bromine generating current (3 mA).

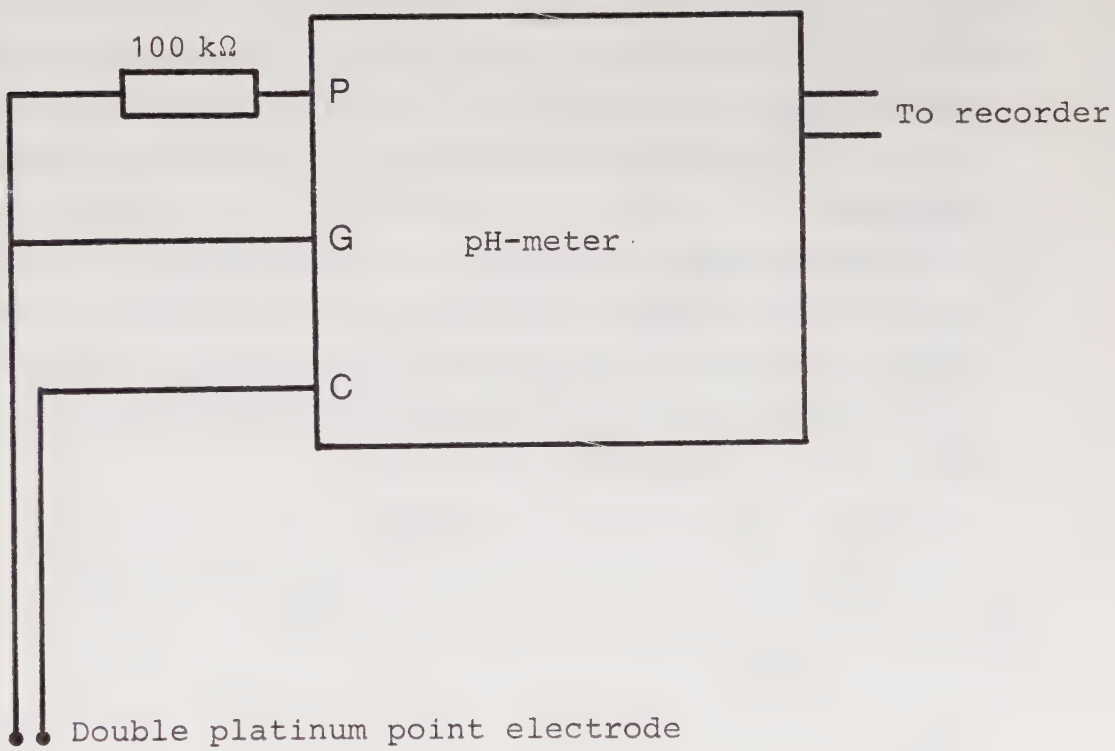


Fig. 4. Schematic representation of the indicating system used in (I) and (II).
P = Polarization terminal
G = Glass terminal
C = Calomel terminal

Results and discussion

Alkylphenols (I). Fifty alkylphenols have been determined by the coulometric bromine titration method. Of these 41 are mononuclear alkylphenols while the remainder are mainly binuclear phenols which from the reactivity point of view are related to the alkylphenols. With two exceptions *monobromination* of mononuclear alkylphenols takes place in media III and IV, containing 40 and 10% water, respectively, and without pyridine (see (I) Table 2). Alkylphenols with alkyl groups in the *meta* position are on the whole more reactive with bromine than other types of alkylphenols due to the cooperation of the *ortho-para* directing influences of the hydroxyl group and the *meta* standing alkyl groups. Accordingly, the less bromination-promoting medium IV generally must be used for their monobromination.

An exception from the previous rule for *meta*-substituted alkylphenols is constituted by phenols with tert.butyl groups in the *meta* positions in that they are less reactive, probably due to steric hindrance.

2,4,6-Trialkylphenols may react with bromine in spite of the absence of free *ortho* and *para* positions. It is believed that a paraquinoid bromocyclohexadienone (V) is formed *e.g.* in the case of 2,4,6-trimethylphenol. The mean relative error for the monobromination of alkylphenols was ± 1.2%.

In the *full bromination* of alkylphenols, 2 or 3 hydrogen atoms in the aromatic ring are exchanged for bromine (see (I) Table 2). In this case a more bromination-promoting medium is necessary. This is because the first entering bromine atom has a deactivating influence. Accordingly, a pyridine-containing medium is nearly always necessary, the only phenol which could be fully brominated in a pyridine-free medium was 3,4,5-trimethylphenol.

The reaction-inhibiting effect of tert.butyl groups is also manifested in the full bromination of alkylphenols. Thus, 3-tert.butylphenol and 3,5-ditert.butylphenol could not be fully brominated in

any of the titration media used in this work. The mean relative error for the full bromination of alkylphenols was ± 1.5%.

The results of the *bromination of some activated alkylphenols*, mainly binuclear are presented in (I) Table 4. The results are not as uniform as for the regular alkylphenols in that the phenols in question are more irregular in their reaction with bromine. Although the monobromination as well as the full bromination method failed in several instances, they never failed for one and the same phenol. Accordingly, all phenols tested could be quantitatively titrated using either the mono- or the full bromination method or both of them. This demonstrates the usefulness of having two bromination methods available.

Deactivated phenols (II). Pyridine-containing titration media must be used throughout for the quantitative bromination of deactivated phenols (see (II) Table 1). No quantitative monobromination is possible for this type of phenol using the standard media. Accordingly, only one bromination method *viz.* the full bromination method, is available. An exception is constituted by certain halophenols, *e.g.* 4-bromo-3,5-dimethylphenol, in which the activating influence of the two alkyl groups exceeds the deactivating influence of the bromo atom. Accordingly, it is an activated phenol and can be monobrominated in a pyridine-free medium as well as fully brominated in a pyridine-containing one.

Although the majority of the compounds could be determined with good accuracy, difficulties were experienced with certain phenols containing *ortho* substituents capable of forming a hydrogen bond with a phenolic hydroxyl group. Thus salicylic acid and *o*-hydroxyacetophenone could not be titrated. Although the former reacted, the results were inaccurate due to incomplete decarboxylation. In this case, the Delgado[13] aqueous medium of pH 3 gave good results with our standard conditions. It is noteworthy that Delgado's[13] medium failed for *o*-hydroxyacetophenone. Another example of a phenolic type which could not be assayed is given

by two iodophenols, namely 2- and 4-iodophenol. For unknown reasons they reacted sluggishly and gave a sloping titration curve which did not permit any determination of the equivalence point. The method as developed is widely applicable for deactivated phenols, the mean relative error being ± 1.2%.

A method for distinguishing between activated and deactivated phenols, and based on the shape of the titration curve in medium III-1, was also developed (see (II) Fig. 1). This method seems to be the first one to discriminate between activated and deactivated phenols.

COULOMETRIC ANALYSIS OF DIHYDRIC PHENOLS AND ASCORBIC ACID (III)

In the third paper a coulometric method is described for the quantitative analysis of easily oxidized organic substances using iodine as an oxidizing agent. The principle of the method is to oxidize the substance with an excess of iodine in a water-acetic acid medium and then titrate the iodide formed with anodically generated silver ion. The titration is followed by dead-stop indication using two platinum electrodes. The method has been applied to the assay of dihydric phenols with hydroxyl groups in the *ortho* or *para* positions and to the determination of ascorbic acid in various pharmaceutical preparations.

1,2- and 1,4-Dihydroxyphenols react to the corresponding quinones in the presence of oxidizing agents such as iodine, *e.g.*

$$\text{Hydroquinone} + I_2 \rightleftharpoons \text{p-Benzoquinone} + 2I^- + 2H^+ \qquad (2)$$

Ascorbic acid reacts in a similar way

$$\text{Ascorbic acid} + I_2 \rightleftharpoons \text{Dehydroascorbic acid} + 2I^- + 2H^+ \qquad (3)$$

Kolthoff[14] studied the influence of pH on the iodine oxidation of hydroquinone and calculated that for a quantitative reaction the pH must be 6 or higher as the equilibrium in equation (2) lies too far to the left at lower pH. Accordingly, the oxidation is favoured by raising pH. However, at higher pH, aerial oxidation of the actual

dihydric phenols can cause low results. There is also a tendency for iodine solutions to produce iodide at higher pH. Hence, it is desirable to perform the oxidation at the lowest possible pH which is compatible with quantitative oxidation.

When performing the titration in the conventional way, *i.e.* either directly by adding iodine up to the end-point or indirectly with back-titration of the excess of iodine, the assay is based on a determination of the iodine consumed.
However, the equations above suggest that there should be another way of evaluating the result of the oxidation, namely by determining the iodide formed. This procedure should have a number of advantages compared to the methods currently in use. Thus, the equilibrium will be displaced to the right by the consumption of iodide ion and the displacement can be further enhanced by using a large excess of iodine. Because of this the titration can be performed at a lower pH than when the assay is based on the iodine consumption, with decreased risk of aerial oxidation.

In the present method the iodide formed is determined by constant-current coulometry using anodically generated silver ion. Accordingly, the method has the general advantage of coulometric methods, *i.e.* no titrants are necessary. The titration can also be performed with rather simple and inexpensive equipment found in most laboratories (see (III) p. 710).

Results and discussion

Appearance of titration curve. A typical titration curve is shown in Fig. 5. It consists of two regions corresponding to depolarization connected by a section corresponding to polarization.

At the beginning of the titration the indicator electrode is depolarized, probably by the I_2/I_3^- - couple. As the iodide ion is consumed by the silver ion, a polarization takes place and the electrode is polarized to a level dependent on the concentration of the acetate ion, since addition of sodium acetate decreases it. This

can be explained by the formation of silver acetate complexes AgOAc and $Ag(OAc)_2^-$ which reduces the concentration of silver ion, controlling the level to which the electrode is polarized. When the production of silver ion is continued the indicator electrode becomes depolarized again. For this depolarization to take place the presence of Ag^+ as well as I_2 seems to be necessary.

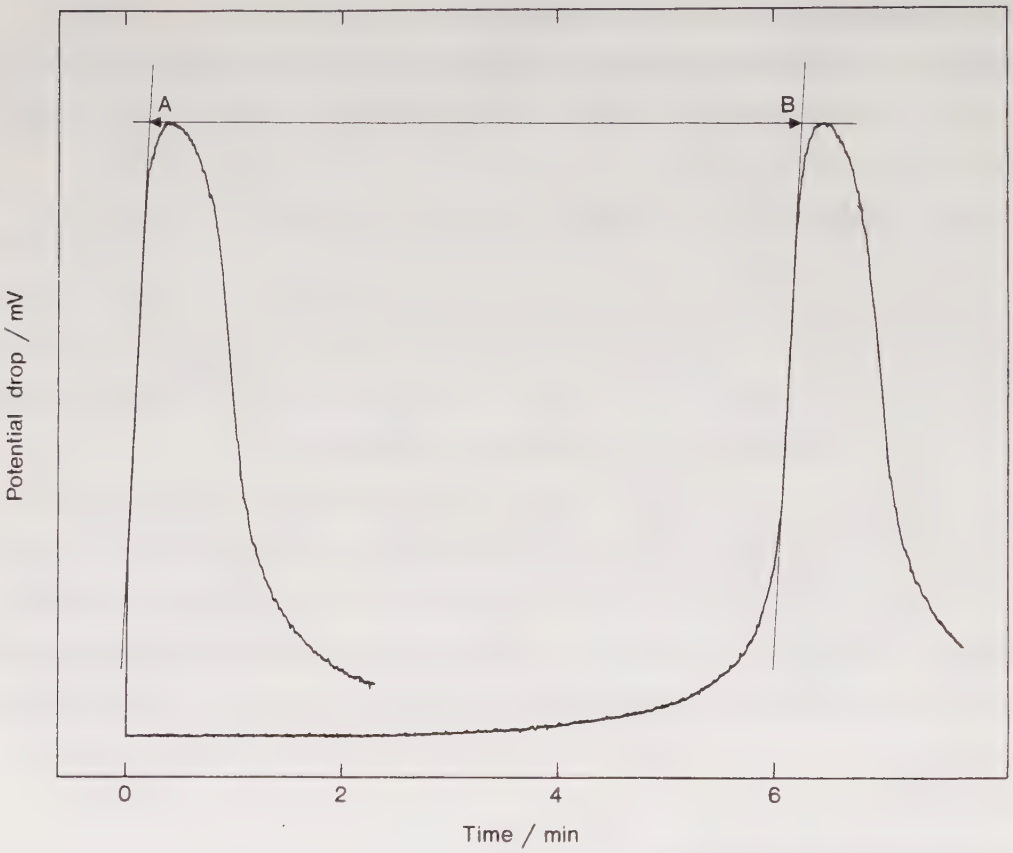


Fig. 5. Titration curve with construction for evaluating the endpoint.

The width and height of the polarization part of the curve give some indication of the reliability of the titration. Ideally the width and height should be the same as in the blank titration. Increased width is a sign of disturbing reactions and if the widening is considerable, the result should be discarded.

Reaction and titration medium. Experiments with aqueous acetic acid indicated that a water content of 40% v/v was suitable and

this mixture has generally been used as reaction and titration medium. Quantitative results were obtained for all but one of the phenols investigated. The exception was 1,2-dihydroxy-4-methylbenzene in which case the apparent pH had to be raised from 1.6 to 2.9 by addition of sodium acetate.

Stability of solutions. Glacial acetic acid is a suitable solvent for the actual dihydric phenols, their solutions being stable for several days. For ascorbic acid, aqueous acetic acid containing 8% v/v acetic acid was used for storage. Ascorbic acid is not stable in this medium, the decomposition amounting to about 10% after 10 hr.

Interfering substances. The vitamin preparations investigated contain other compounds besides ascorbic acid, *e.g.* sugars of various kinds, chloride and iron in the form of Fe^{2+}. Of these only Fe^{2+} was found to interfere significantly. However, it was easily removed by pouring the solution through a cation-exchanger in acid form before titration. The presence of chloride up to 5% of the amount of iodide did not influence the results.

Results. The mean relative error is $\pm$ 1.0% for the 8 phenols tested and the relative standard deviation lies in the range 0.2 - 1%. For the vitamin preparations the results were compared with those obtained by a pharmaceutical laboratory, using official methods. The agreement was surprisingly good considering the fact that the two analyses were performed on separate tablets from the same batch. It can be concluded that the special advantages of the method are the elimination of titrant solutions, easy evaluation of the end point and the possibility of performing the oxidation at relatively low pH, thereby decreasing the risk of aerial oxidation (see also (III) Tables 1 and 2).

ANALYSIS OF PHENOLS AND RELATED SUBSTANCES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) (IV-VII)

In papers (IV, V) the retention behaviour of alkylphenols (IV) as well as brominated alkylphenols (V) is studied using four liquid

chromatographic columns: three straight phase systems on nitrile phases and one reversed phase system on octadecylsilane. Product formation in the coulometric bromination of some alkylphenols were also studied (V). In paper (VI) an analytical method based on HPLC with electrochemical detection for the determination of two catecholic amino acids (5-S-cysteinyldopa and dopa) (see Fig. 1) in human serum is described. The possibilities of the simultaneous analysis of several biologically important catechols in human serum and urine by means of HPLC and electrochemical detection are evaluated in (VII).

Development of HPLC

High performance liquid chromatography (HPLC) is a recently developed technique based on classical column liquid chromatography. Great advances have been made in the technology of the equipment involved and this trend is continuing. In HPLC separations a mobile phase is pumped at pressures up to 700 atm through a chromatography column. The columns are usually made of precision-bore stainless steel. The packing materials have been improved to such an extent that separation efficiencies now approach those of gas chromatography.

The column effluent is passed through a special detector system the response of which is usually displayed on a strip-chart recorder.

The basic components of liquid chromatography are shown in Fig. 6.

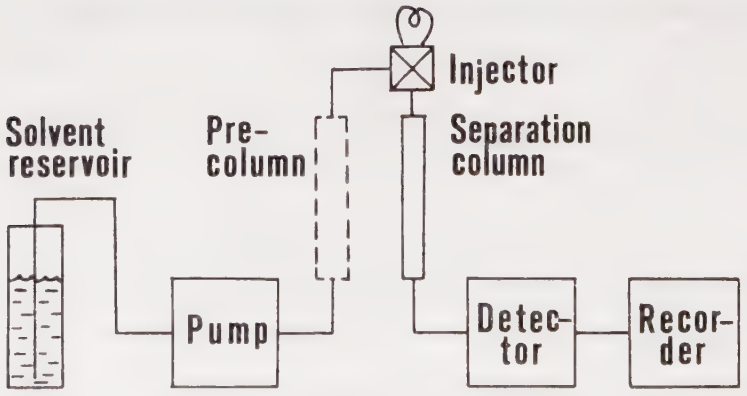


Fig. 6. Equipment for HPLC.

HPLC is today one of the most important analytical techniques and this is partly due to the fact that the other dominating separation method, gas chromatography (GC), often cannot be used for the separation of highly polar, thermally instable or involatile substances. In these cases HPLC is the ideal separation technique and its future potential use in the laboratory is considered to be even greater than that of GC.

The problems encountered in classical LC, utilizing an organic phase physically held on the surface of an inorganic carrier, have been circumvented in *bonded phase liquid chromatography (BPLC or BPC)*. Here the organic phase is chemically bonded to the carrier, generally silica, and is thus insoluble in the mobile phase. Although chemically bonded phases for LC were used as early as 1950[15], the interest in BPLC was small until the late sixties and early seventies when especially Aue *et al.*[16,17,18] developed methods for preparing chemically bonded phases. In fact, their attempts originally aimed at preparing chemically bonded phases for GC but the greater potential value of these phases for LC was soon realized. Among other important contributers to the science of BPLC are Stewart and Perry[19], Kirkland and DeStefano[20], Parr and Novotny[21] and Majors and Hopper[22]. Stewart and Perry seem to have been the first ones to prepare a reversed phase packing material for LC with chemically bonded octadecyl groups, a packing material which today is among the most used in HPLC. Recent developments in this field are covered by Majors[23].

Bonding of the organic phase to silica is usually brought about by reacting organochloro- or organoalkoxysilanes with the surface silanol groups (Si-OH) of silica, whereby a stable siloxane bond (Si-O-Si-C) is formed. Depending on the kind of organosilane and reaction conditions, either a monomeric or polymeric type of siloxane phase results. Today microparticulate packing materials (5 - 10 µm) are almost exclusively used for analytical separations. Chemically bonded phases are available with a wide range of functionalities like NH_2, CN, Ph, C_2-C_{18} alkyl groups and ion-exchan-

ging groups. Of these the alkyl type phases, and especially the C_{18} phase, are the most frequently used. It is estimated that more than 60% of the HPLC separations today are made on chemically bonded alkylsilane phases, and the present trend indicates a further growth of this technique[24]. The main reason for this is the availability of a variety of such packing materials of good quality and the possibility of separating polar as well as nonpolar compounds by changing the composition of the mobile phase[25,29]. Thus, separations that previously were performed using silica or ion exchangers can now with advantage be carried out on alkylsilane phases. The success of HPLC as an analytical tool is closely connected with the development of chemically bonded phases. Some important advantages of these phases in comparison with those previously used are thermal and solvolytic stability together with insolubility in solvents and rapid equilibration with the eluent which permits troublefree gradient elution[30].

Column packing is an important part of the HPLC technique. The decrease of the particle size of the packing material down to 5 - 10 µm has necessitated an exchange of the older dry packing technique for new slurry packing methods[31,32,33]. It has been pointed out by Bristow[30] that the process of packing the microparticulate separation material into the column is the subject of much mystique. This fact has made the researchers in the HPLC field too much dependant on expensive factory-made columns.

In order to improve this situation a simple column packing technique based on the idea of upward slurry packing[34] is described in (VII), which has been found to be extremely reliable for both 5 and 10 µm hydrocarbonaceous packing materials[28]. The technique is also applicable to silica and to normal phase chemically bonded material *e.g.* nitrile and amino phases[28].

Even the best column can be spoiled by extra column effects due to incorrect connection of injector and detector to the column[35]. A convenient way of avoiding such effects is described in (VII),

where a piece of narrow bore stainless steel tubing is used for connection on the injector side and a piece of teflon tubing on the detector side (see Fig. 7).

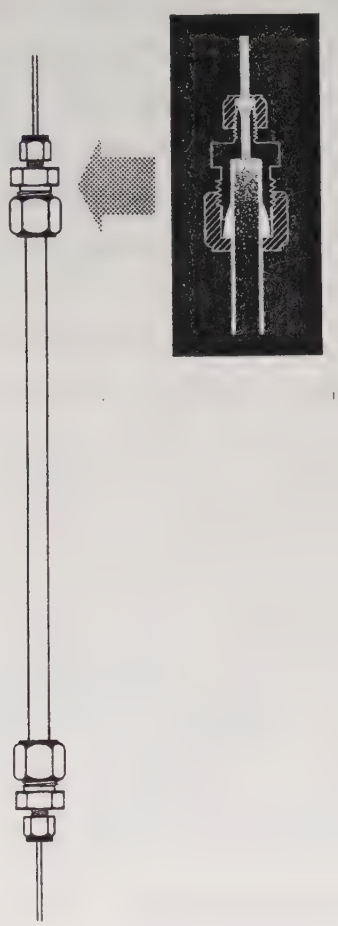


Fig. 7. Column and design of column head and bottom for valve injection. Very thin stainless steel mesh discs are placed at both ends of the column.

Detectors for HPLC are generally more or less specific *e.g.* the ultra-violet (UV) detector, the fluorescence detector and the refractive index (RI) detector. Of these the first one has been utilized in this work (IV-VII). Although there is a need in HPLC for more general detectors than these, a number of approaches have also been made to develop new specific detectors. One of the most interesting new detectors is the electrochemical detector developed by Kissinger[36] which in this work has proved to be ideally suited for the detection of catecholic compounds in biological

fluids (VI,VII). Neither UV nor fluorescence detectors can be used in this case because of the low concentration of catecholic compounds in urine and serum.
The electrochemical detector used in this work is operated in the three-electrode mode. A conventional potentiostatic system is used with an auxiliary and a reference electrode (Ag/AgCl) together with the working electrode which is made of carbon paste and set in the flow stream emerging from the column. The electrode surface is part of the channel wall formed by sandwiching a fluorocarbon gasket between two blocks machined from plexiglass or Kel-F. The working electrode is held at a fixed predetermined potential to oxidize (or reduce) electroactive components passing by.

Recently Kissinger[36] has discussed the scope and limitations of electrochemical detection and pointed out that complex surface reactions are involved and some effort is required to simultaneously optimize both column and detector performance. A very fortunate bonus is the fact that reversed phase chromatography is ideally suited for electrochemical detection[37]. Furthermore the electrochemical detection of catechols[37] is advantageous due to their low oxidation potential (VI,VII).

Theoretical considerations

Most of the work done to explain the retention mechanism in bonded phase chromatography has been directed towards nonpolar phases. An understanding of reversed phase LC requires an appreciation of solution phenomena in water. Chromatography on hydrophobic stationary phases, *e.g.* of the C_{18} type, is most often performed using a mobile phase consisting of water mixed with a polar organic solvent. The role of the solvent is fairly clear. Locke[38] has suggested that the relative retention for two closely related solutes may in certain cases be determined solely by the difference in solubility between the two components in the mobile phase.

The mechanism of retention on the stationary phase is open to question. Karger *et al.*[39] discussed the role of hydrophobic effects in

reversed phase LC in terms of hydrophobic selectivity relative to dispersion forces. Hydrophobic effect is a term frequently used to indicate cavity formation of water molecules and non-polar clustering, involving the expulsion of non-polar parts of organic molecules from water. It was shown that the hydrophobic selectivity can be accounted for by using a new topological index, called molecular connectivity, which is proportional to the cavity surface area. This index is quite simple to calculate and is used for estimating non-polar group contributions to retention in reversed phase LC.

A more detailed theory for the interaction between solutes and hydrocarbonaceous bonded stationary phases, using water and mixtures of water and organic solvents as mobile phases, has been presented by Horvath *et al.*[40] They consider the surface of the reversed phase packing material as a "molecular fur" of covalently bonded hydrocarbon chains. In order to describe non-polar interactions in polar solvents they adopted the so-called solvophobic theory, originally put forward by Sinanoglu[41]. This theory treats the processes in terms of properties of the bulk solvent, such as surface tension and dielectric constant and in terms of solute properties such as surface area and dipole moment.

The interaction between the solute and the stationary phase in solvophobic chromatography is considered a reversible association of the solute molecules, S, with the hydrocarbonaceous ligand, L, at the surface.

$$S + L \rightleftharpoons SL \qquad (4)$$

The complex SL is assumed to be formed by solvophobic interactions which can be visualized according to Fig. 8.

The equilibrium constant, K, for this process is related to the capacity factor by the following equation

$$k' = K \phi \qquad (5)$$

ϕ = volume ratio of the stationary and mobile phase.

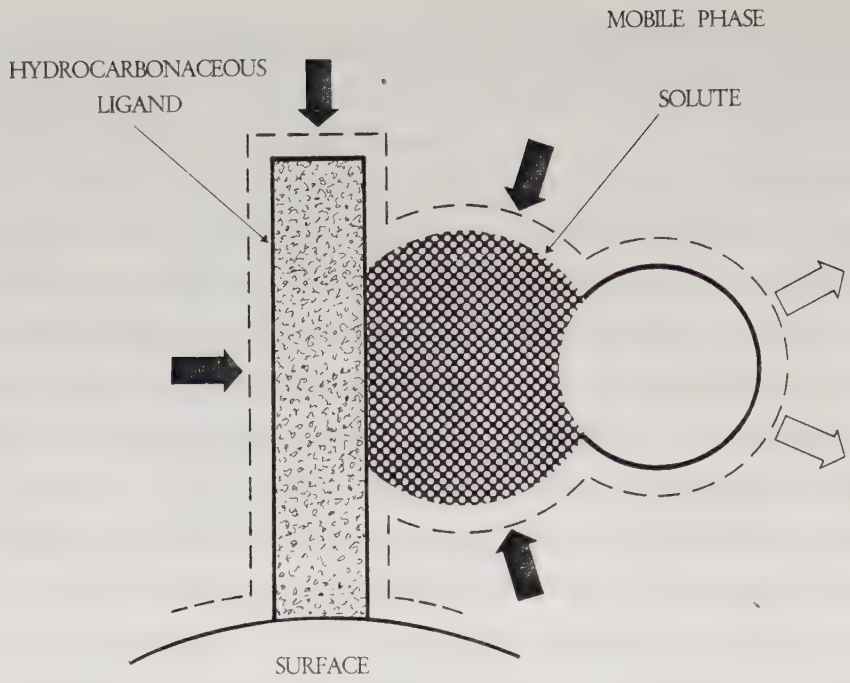


Fig. 8. Association between the solute and non-polar ligand at the surface of the stationary phase in reversed phase chromatography. Solid arrows indicate forces facilitating complex formation which are counterbalanced by attractive interactions between solute and solvent (open arrows).
◯ = polar part; ⊛ = non-polar part

The relationship between K and the difference in the free energy change of the association process (ΔG) is expressed by

$$\ln K = - \frac{\Delta G}{RT} \qquad (6)$$

R is the gas constant and T is the absolute temperature.

For unionized solutes Horvath *et al.*[40] evaluated K, employing the solvophobic theory, whereby an expression for the capacity factor in terms of macroscopic solute and solvent properties was obtained. In cases where pure water is used as mobile phase, the chromatographic process is governed by the previously mentioned hydrophobic effect.

According to Horvath *et al.*[40], for closely related substances the capacity factor (k') can be related to the molecular dimensions of the solute at fixed eluent composition

$$\ln k' = A'' + \frac{N}{RT} \cdot \Delta A \cdot \gamma \qquad (7)$$

A'' = constant

ΔA = the contact surface area of the associated species

N = Avogadro's number

R = the gas constant

T = the absolute temperature

γ = surface tension

The linear relation according to equation (7) was experimentally verified by Horvath *et al.*[40] which implies that the contact area between the solute and the ligand is proportional to the hydrocarbonaceous surface area of the substance investigated.

For members of a homologous series the carbon number is proportional to the molecular surface area and thus a linear relationship should be obtained when plotting the logarithm of the capacity factor against the carbon number. Such a dependence was also observed and is exemplified in Fig. 9 (IV) wherein log k' is plotted *vs.* the carbon number for normal alcohols.

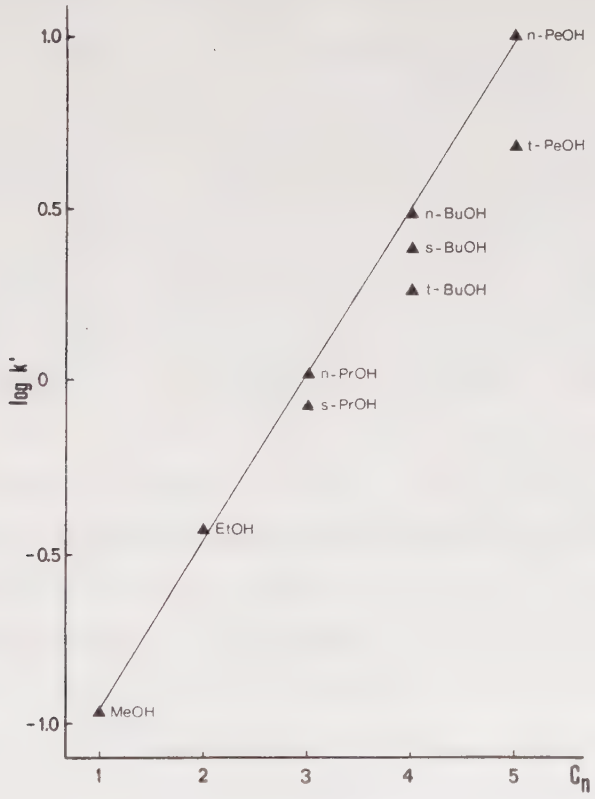


Fig. 9. Log k' *vs.* carbon number for aliphatic alcohols. Column: μBondapak C_{18}. Eluent: methanol-water, 5:95 (v/v) (IV).

In most cases, the eluent used with bonded non-polar phases are mixtures of water and organic solvents as modifiers, the most popular ones being ethanol and acetonitrile (IV, V, VI). Retention can easily be controlled by varying the amount of modifier (V), retention being highest with pure water. The observed decrease of the capacity factor can largely be attributed to the concomitant decrease in surface tension[40] (V).

For ionizable substances it has been shown that pH and ionic strength can be used to control retention in reversed phase chromatography, using neat aqueous eluents[42] (VII). The effect of solute ionization and ionic strength on the retention of weak acids, bases and ampholytes was investigated theoretically in the light of the solvophobic theory and using a semi-empirical extension of the Debye-Hückel theory[26].

A commercially available reversed phase packing material was used for experimental testing of the theory. Good agreement was obtained[26].

In (VII) the effect of ionic strength on the retention of catecholic amino acids was investigated for four commercially available reversed phase ODS packing materials by adding sodium sulphate to an aqueous mobile phase containing 2.9 g phosphoric acid per litre, (see Fig. 10). As can be seen, a considerable difference between the k'-values of the test substances were obtained. In Table 2 the names of the packing materials and some properties are given. It is argued that under otherwise fixed conditions the intrinsic retentative capability is higher for packing materials with higher carbon content. This is not verified by the results obtained here with salt-free systems in that the highest k'-values are observed for the low-carbon phase Spherisorb ODS. The considerable difference between Partisil ODS and Spherisorb ODS is also noteworthy. Furthermore, it is evident that there is no correlation between retention and silanol interaction, see Table 2.

The change in retention observed for Spherisorb ODS and to a smaller extent for Partisil ODS is in accordance with the theory of

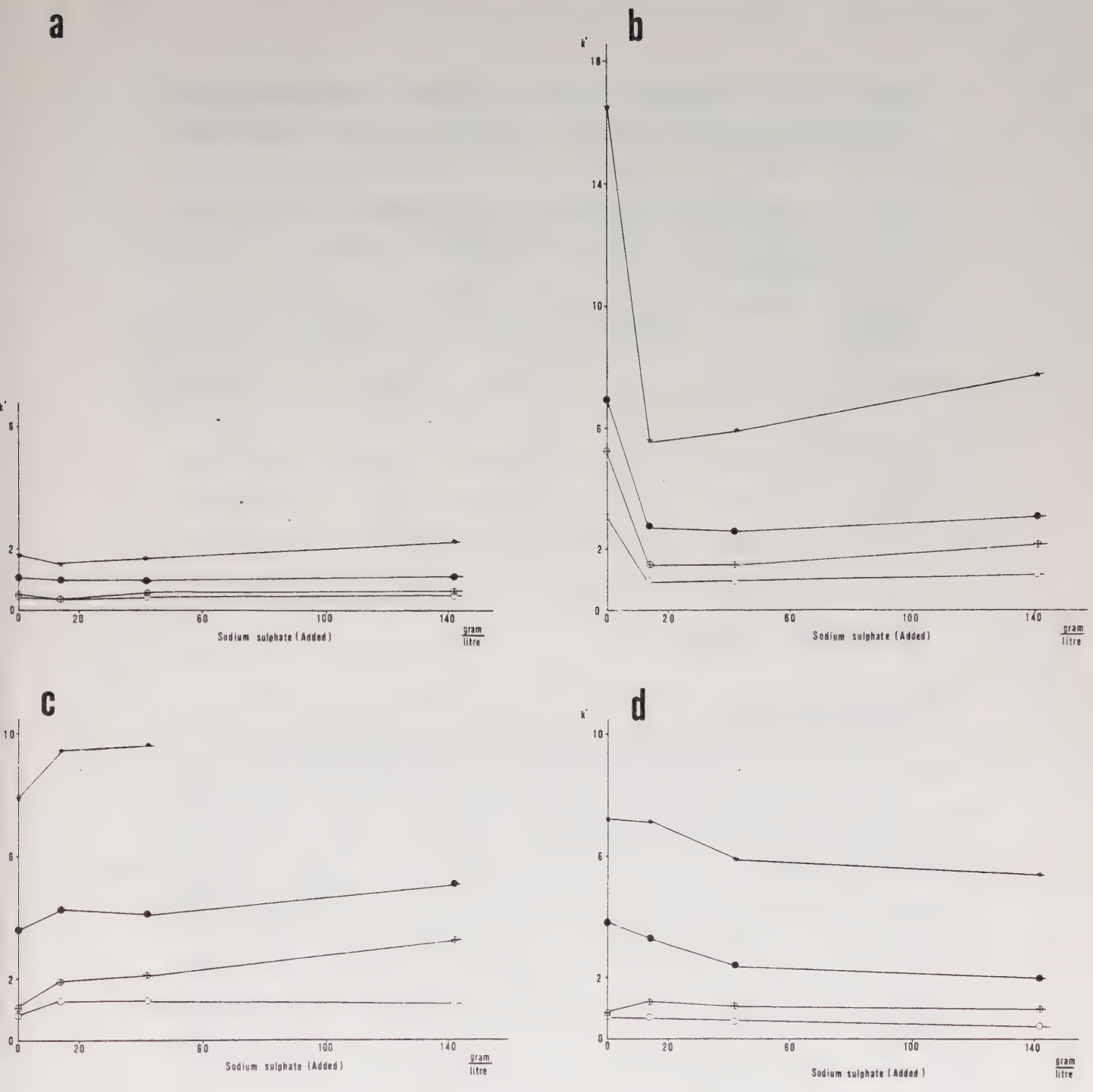


Fig. 10. Relationship between capacity factor, k', and amount of sodium sulphate added to the mobile phase.
Column: Reversed phase ODS materials (10 µm), 200 x 5 mm.
Eluent: Water, 2.9 g phosphoric acid per litre, with the addition of sodium sulphate. Flow rate: 100 ml/h.
✱ 5-S-cysteinyldopa; ● dopa; ○ 2-S-cysteinyldopa; ⊕ 2,5-S,S-dicysteinyldopa. a) Partisil ODS; b) Spherisorb ODS; c) Nucleosil C_{18}; d) LiChrosorb RP-18.

Horvath *et al.*[26] However, for Nucleosil C_{18} and LiChrosorb C_{18} the retention behaviour is not in accordance with the theory.

Table 2. Carbon content, surface area and retention characteristics for different chemically bonded reversed phase materials.

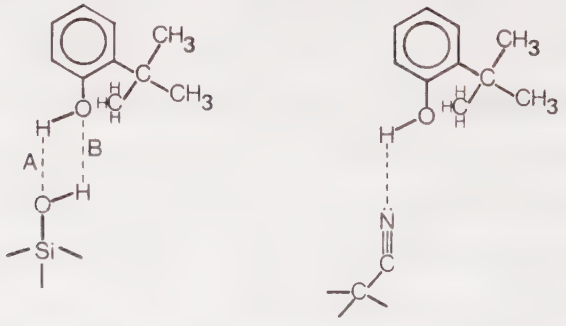
Column packing material (10 µm)	Carbon content %	Surface area* m^2/g	k' with hexane as eluent	
			benzene	nitrobenzene
Partisil ODS	4.00	300	0.15	0.80
Spherisorb ODS	6.67	220	0.14	0.58
Nucleosil C_{18}	18.6	300	0.20	0.45
LiChrosorb RP-18	19.8	400	<0.1	0.21

*According to manufacturer

Alkylphenols (IV)

The retention behaviour of a series of alkylphenols was investigated using three straight phase systems (nitrile phases) and one reversed phase system (ODS-bonded phase). On the nitrile phases the alkylphenols are eluted in the order: di-, mono- and non-*ortho*-substituted phenols which is primarily due to decreased steric hindrance to hydrogen bonding in the same order.

The reversed elution order of 2,6-dimethyl- and 2-tert.butylphenol on silica and nitrile phases, respectively, can be explained by the difference in hydrogen bond interaction:



The surface silanol groups are capable of forming hydrogen bonds with both the oxygen and the hydrogen atoms of the phenolic hydroxyl

group although the former interaction (marked B in the figure) is the one generally considered in the literature. The cyano group, on the other hand, is only capable of forming a hydrogen bond to the hydrogen atom of the phenolic hydroxyl. As indicated by the figure, a bulky *ortho* substituent, *e.g.* tert.butyl, should interfere to a greater extent with the dominating B-type hydrogen bonding to the silanol group than with the hydrogen bonding to the cyano group. Accordingly, 2-tert.butylphenol is eluted earlier than 2,6-dimethylphenol on silica, while the opposite is valid on a nitrile phase.
Low surface coverage on a chemically bonded phase (Cyano Sil-X-I), as compared to highly loaded LLC nitrile columns, increases the contribution to retention by adsorption to remaining silanol groups, making the elution order more similar to that on silica.

Application to analysis

Combination of straight and reversed phase LC. In Fig. 11, log k' for the reversed phase LC system is plotted against log k' for a straight phase system, Cyano Sil-X-I. All of the phenols investigated are clearly divided into the three structure classes. Accordingly, it appears to be possible to recognize di-, mono- and non-*ortho*-substituted phenols by combining straight and reversed phase chromatography. Another characteristic of the two-column plots in Fig. 11 is that within each structure group the points are arranged according to alkyl carbon number. Thus, starting from the top of each structure line, the alkyl carbon number will decrease along the line. Points that represent phenols with the same alkyl carbon number trend to cluster, which makes identification of individual phenols difficult. For a complete separation of these phenols, we used capillary column GC. However, it should be pointed out that it ought to be possible to achieve a better resolution of the points in Fig. 11 by optimizing the composition of the mobile phase for certain phenols.

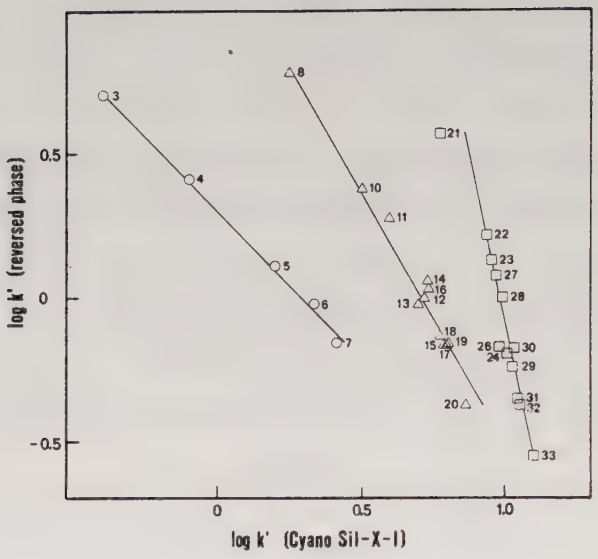


Fig. 11. Two-phase plot for alkylphenols. ○di-*ortho*-, △ mono-*ortho*-, □non-*ortho*-substituted phenols.

Brominated alkylphenols (V)

The fifth paper describes the application of HPLC to the analysis of product formation at coulometric bromination of alkylphenols. The combination of HPLC and coulometric bromination by the method described in (I) offers a unique possibility for the study of retention characteristics of brominated phenols since a practically unlimited number of *ortho*- and *para*-brominated phenols can be readily synthesized. The main bromination reaction is exchange of hydrogen for bromine in the free *ortho* and *para* positions and the coulometric method generally yields one unambiguous product for the phenols investigated. The study concerns three types of alkylphenols: 4-alkylphenols and 2,4- and 2,6-dialkylphenols. The brominated products are chromatographed by straight and reversed BPC.

For all phenols investigated the introduction of bromine results in an increase of the retention on the reversed phase system, independently of whether the substitution takes place in an *ortho* or *para* position. For each group of phenols there is a linear relationship in the reversed phase system between the capacity factor for the brominated (k'_2) and non-brominated (k'_1) phenols, *i.e.*

$$k'_2 = m \cdot k'_1 + b \qquad (8)$$

The linear correlation to eqn. (8) for 2,6-dialkylphenols, 4-alkylphenols and 2-bromo-4-alkylphenols is summarized in Table 3.

Table 3. Linear correlation (r) between k'-values of brominated (k'_2) and non-brominated (k'_1) phenols. Column: μBondapak C_{18}. Eluent: ethanol/water 60:40, v/v.

Compounds	$k'_2 = mk'_1 + b$			
	m	b	r	n
2,6-Dialkylphenols	1.30	0.43	0.9990	7
4-Alkylphenols*	1.22	0.19	0.9998	7
2-Bromo-4-alkylphenols*	1.43	0.15	0.9997	7

*Values for 4-cyclohexylphenol and 2-bromo-4-cyclohexylphenol omitted.

It follows from eqn. (8) that the separation factor, α, is a hyperbolic function of k'_1.

$$\alpha = \frac{k'_2}{k'_1} = m + \frac{b}{k'_1} \qquad (9)$$

which has the asymptot $\lim_{k'_1 \to \infty} \alpha = m$. Thus it can be expected that for alkylphenols with large k'-values (high alkyl carbon number) the separation factor, α, between the brominated and non-brominated phenol approaches the asymptotic value of m (see Table 3). Plots of α *vs.* k'_1 for the phenols in Table 3 are shown in Fig. 12.

Deviation from linearity was observed when k'_2 was plotted *vs.* k'_1 for 2,6-dimethylphenol, 2-bromo-4-methyl- and 2-bromo-4-ethylphenol (V) and this is also reflected in the deviation from the hyperbolic function in Fig. 12 for these phenols.

The α-values for the phenols with the largest alkyl carbon number (or the highest k'-values) are for the different groups: α = 1.40 for 2,6-di-tert.butylphenol, 1.29 for 4-octylphenol and 1.48 for

2-bromo-4-octylphenol. These values compare favourably with the slopes (m) in Table 3.

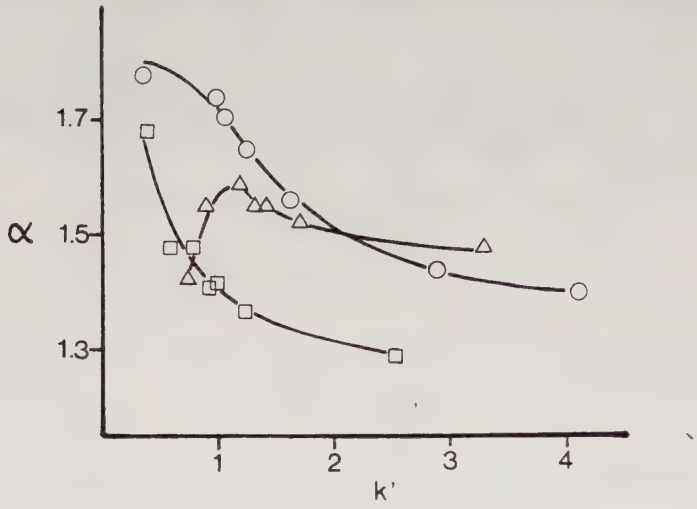


Fig. 12. Separation factor, α, *vs.* k'_1, for brominated/non-brominated alkylphenols on μBondapak C_{18}. Eluent: ethanol/water 60:40. ○ 2,6-Dialkylphenols; □ 4-Alkylphenols; △ 2-Bromo-4-alkylphenol.

In the straight phase LC-system introduction of bromine into the *ortho* position of 2,4-dialkyl- and 4-alkylphenols results in a marked decrease in retention due to decreased strength in the hydrogen bonding to the stationary phase caused by steric hindrance of the phenolic hydroxyl group. Introduction of bromine into the *para* position of 2,6-dialkylphenols causes an increase in retention with a linear correlation of 0.999 between k' for brominated and non-brominated phenols.

The combination of coulometric bromination and liquid chromatography can be used for separation of isomeric alkylphenols, which are difficult to separate as such by chromatographic methods, *e.g.* 3- and 4-methylphenol.

Quantitative determination of 5-S-cysteinyldopa and dopa in serum (VI)

The concentration of 5-S-cysteinyldopa (see Fig. 1), the major isomer of the phaeomelanin precursors, has been determined rou-

tinely with a fluorometric technique for some years in urine from melanoma patients[6]. The amount of 5-S-cysteinyldopa excreted gives information on the degree of dissemination of the tumour.

The fluorometric method is relatively simple but not without drawbacks since certain drugs such as salicylates cause interference. Furthermore, the sensitivity is not great enough to measure 5-S-cysteinyldopa in blood, except in advanced melanoma cases[5]. Thus a more sensitive method for determining cysteinyldopa and other catechols is called for. In this work a method is described for quantitative determination of 5-S-cysteinyldopa and dopa in serum involving high performance liquid chromatography and electrochemical detection. The chromatographic system allows estimation of injected amounts corresponding to 25 pg of 5-S-cysteinyldopa and dopa. In normal subjects the mean serum 5-S-cysteinyldopa concentration was 28 ng/ml range (0.4 - 2 ng/ml) and the mean serum dopa concentration 6.3 ng/ml (range 4 - 10 ng/ml). Patients with melanoma metastases showed increased serum concentrations of 5-S-cysteinyldopa.
Fig. 13 shows chromatograms obtained from serum from a healthy subject and from a patient with melanoma, illustrating the ease with which 5-S-cysteinyldopa and dopa are detected.

It is estimated that the new method presented here is roughly 1,000 times more sensitive than the fluorimetric method previously used[6]. High selectivity is obtained by purification of serum on alumina before HPLC. Interfering substances are removed on alumina which is a necessity for the use of the electrochemical detector.

Analysis of catecholic amino acids and catecholamines in serum and urine (VII)

This work is an extension of the one presented in the previous paper. HPLC with a chemically bonded reversed phase packing material combined with electrochemical detection has been used to separate and detect the catecholic amino acids dopa, 2-S- and 5-S-cysteinyl-

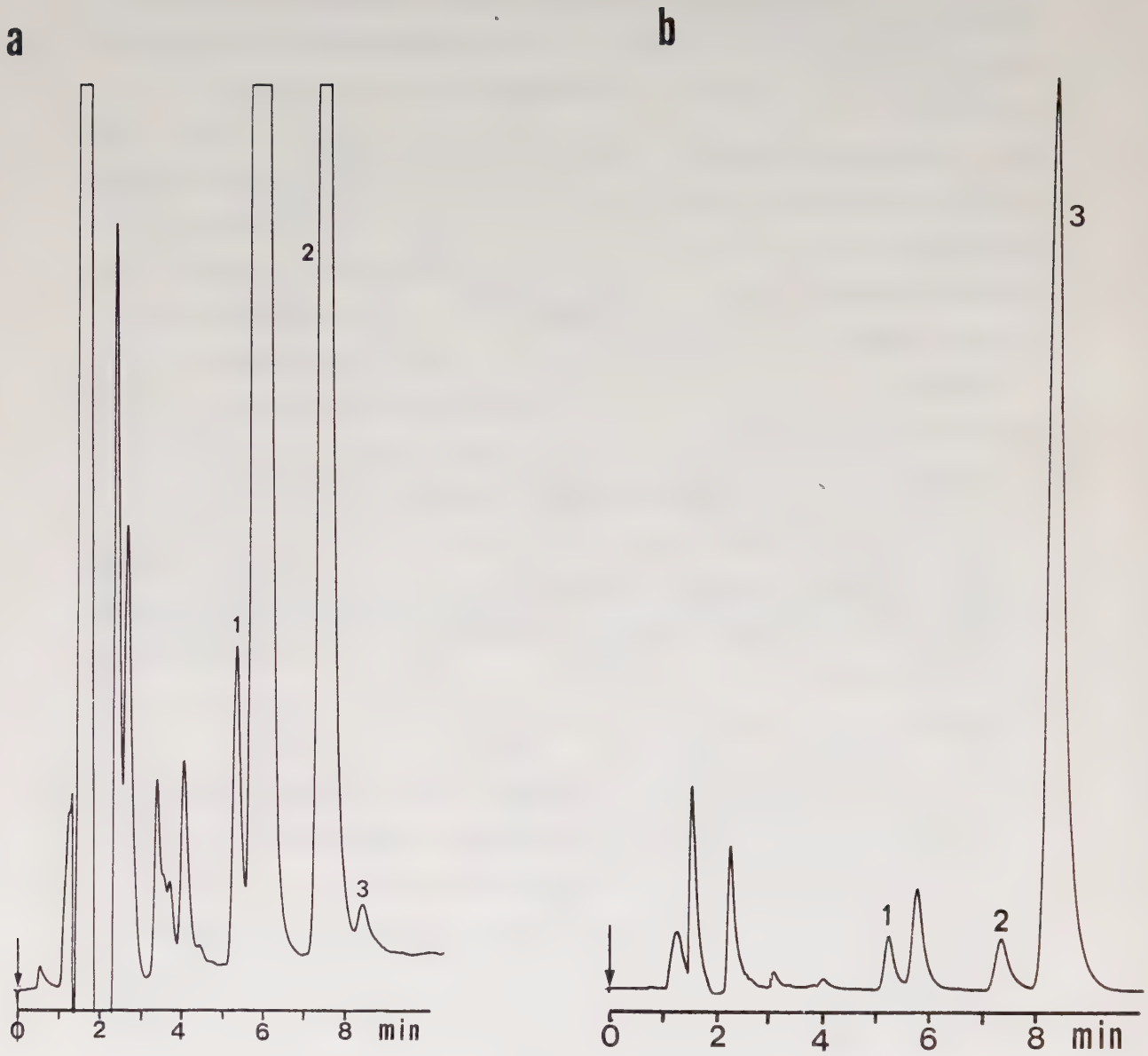


Fig. 13. Chromatograms of purified serum samples from the analysis of 5-S-cysteinyldopa and dopa. The injected amount of isoproterenol (internal standard) is the same in both runs. Peak identity: 1 = dopa, 2 = isoproterenol, 3 = 5-S-cysteinyldopa. Mobile phase: 0.5 % MeOH v/v in water with 2.9 g H_3PO_4 per litre. Column: Nucleosil ODS 5 µm. Flowrate: 100 ml/h. Sample volume: 100 µl.

a) Serum from a healthy person. Peak 3 corresponds to 25 pg 5-S-cysteinyldopa.

b) Serum from a melanoma patient.

dopa and 2,5-S,S-dicysteinyldopa in serum from patients with malignant melanoma (see Fig. 14 a). In urine the catecholamines dopamine, noradrenaline and adrenaline were also separated, as well as the above-mentioned compounds, in a single chromatographic run (see Fig. 14 b). Separation is achieved with the addition of methanesulphonic acid to an acidified aqueous mobile phase. By changing the pH of the mobile phase and the concentration of methanesulphonic acid, the retention can be varied, thus allowing for the optimization of the separation conditions. Modifiers with a longer lipophilic group could also be valuable in cases where a further enhancement of retention is desired, as shown by Horvath *et al.*[25]

A comparison of the performance of four commercial reversed phase packing materials containing chemically bonded octadecyl groups using a standard mixture of catecholic amino acids shows that there is a considerable difference between packing materials from different manufacturers. This was also evident from a study of the variation of retention of the test mixture when the ionic strength of the mobile phase was changed by the addition of sodium sulphate.

Efficiency is markedly increased using 5 µm packing material. The addition of an organic modifier also improves efficiency, presumably due to an enhancement of mass transfer between the phases.

Systems involving combination of reversed phase microparticulate packing materials with acidified aqueous mobile phases containing salts or organic modifiers are well suited for the analysis of catecholic compounds with electrochemical detection. It is possible to detect very small amounts of catecholic amino acids and amines, *e.g.* 25 pg of 5-S-cysteinyldopa. Essential for this result is the selective adsorption of catecholic compounds on alumina prior to separation by HPLC. By this methodology the overall sensitivity and selectivity is considerably improved.

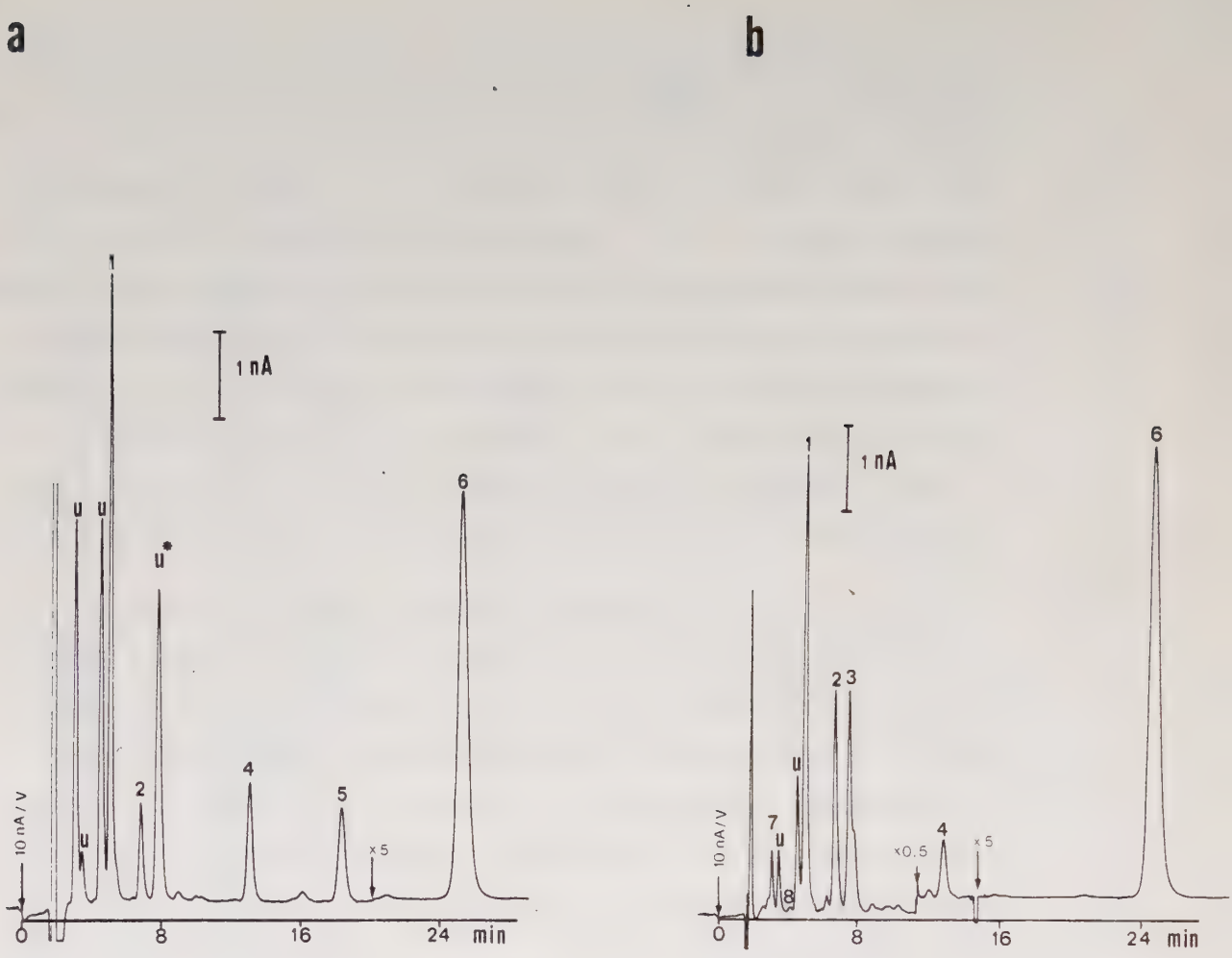


Fig. 14. Chromatograms of purified serum (a) and urine (b) obtained from patients with melanoma metastasis. Eluent: Water, 2.9 g phosphoric acid and 6 g methanesulphonic acid per litre. pH was adjusted to 1.75 with 5 M sodium hydroxide. Column: Nucleosil C_{18} (5 µm), 200 x 5 mm. Flow rate: 102 $ml \cdot h^{-1}$.

Peak identity: 1 = 2-S-cysteinyldopa; 2 = 2,5-S,S-dicysteinyldopa; 3 = dopamine; 4 = dopa; 5 = isoprenaline (internal standard); 6 = 5-S--cysteinyldopa; 7 = noradrenaline; 8 = adrenaline; u = unidentified. 6-S-cysteinyldopa is not detected, retention time between peaks 2 and 3.

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*In contrast to peak 3 in Fig. 14 b, U does not correspond to dopamine which was shown by chromatographing with other mobile phases.

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